Substituting (x) into (viii) gives

$$[\text{XSc}^{\circ}] = [\text{Sc}] + [\text{ScH}^{+}] + [\text{ScH}_2^{++}] + \bar{n} [\text{M}^{\circ}]. \quad (\text{xi})$$

Now, in the Mass Action equations (xii),

$$K_a = \frac{[\text{H}^{+}][\text{A}^{-}]}{[\text{HA}]}, \text{ and } K'_a = \frac{[\text{H}^{+}][\text{HA}]}{[\text{H}_2\text{A}^{+}]}, \quad (\text{xii})$$

which describe the ionization of a dibasic acid, $[\text{A}^{-}]$ corresponds to $[\text{Sc}]$, $[\text{HA}]$ to $[\text{ScH}^{+}]$, etc. Hence

$$[\text{ScH}_2^{++}] = \frac{[\text{H}^{+}]^2 [\text{Sc}]}{K_a K'_a} \text{ and } [\text{ScH}^{+}] = \frac{[\text{H}^{+}][\text{Sc}]}{K_a}. \quad (\text{xiii})$$

Substituting (xiii) into (xi) gives

$$[\text{XSc}^{\circ}] = [\text{Sc}] + \frac{[\text{H}^{+}]^2 [\text{Sc}]}{K_a K'_a} + \frac{[\text{H}^{+}][\text{Sc}]}{K_a} + \bar{n} [\text{M}^{\circ}], \quad (\text{xiv})$$

rearrangement of which gives

$$\bar{n} = \frac{[\text{XSc}^{\circ}] - [\text{Sc}] \left\{ \frac{[\text{H}^{+}]^2}{K_a K'_a} + \frac{[\text{H}^{+}]}{K_a} + 1 \right\}}{[\text{M}^{\circ}]} \quad (\text{xv})$$

$$= \frac{[\text{XSc}^{\circ}] - \alpha [\text{Sc}]}{[\text{M}^{\circ}]} \quad (\text{xvi})$$

Where three ionizing groups are present, the term

$$\frac{[\text{H}^{+}]^3}{K K'_a K''_a}$$

is added to α .

B. Derivation of equation for $[\text{Sc}]$

The concentration of free and combined hydrogen ions $[\text{XH}^{+}]$ can be expressed in two ways, (xvii) and (xviii):

$$[\text{XH}^{+}] = [\text{H}^{+}] + [\text{ScH}^{+}] + 2[\text{ScH}_2^{++}] - [\text{OH}^{-}], \quad (\text{xvii})$$

$$[\text{XH}^{+}] = [\text{XSc}^{\circ}] + [\text{HCl}] - [\text{KOH}], \quad (\text{xviii})$$

where $[\text{HCl}]$ and $[\text{KOH}]$ equal the concentrations which these reagents would reach if run into the same volume of pure water. Combining (xvii) and (xviii),

$$2[\text{ScH}_2^{++}] + [\text{ScH}^{+}] = [\text{XSc}^{\circ}] + [\text{HCl}] - [\text{KOH}] - [\text{H}^{+}] + [\text{OH}^{-}]. \quad (\text{xix})$$

Substituting terms from equation (xiii), and rearranging,

$$[\text{Sc}] = \frac{[\text{XSc}^{\circ}] + [\text{HCl}] - [\text{KOH}] - [\text{H}^{+}] + [\text{OH}^{-}]}{2 \frac{[\text{H}^{+}]^2}{K_a K'_a} + \frac{[\text{H}^{+}]}{K_a}} \quad (\text{xx})$$

$$= \frac{[\text{XSc}^{\circ}] + [\text{HCl}] - [\text{KOH}] - [\text{H}^{+}] + [\text{OH}^{-}]}{\beta}. \quad (\text{xxi})$$

$[\text{HCl}]$ and $[\text{KOH}]$ are alternative terms, depending on which reagent was used in the titration. In general $[\text{OH}^{-}]$ becomes significant only above pH 10. Where three ionizing

groups are present, the term $\frac{3[\text{H}^{+}]^3}{K_a K'_a K''_a}$ is added to β .

Photometric Micro-analytical Estimation of Acetic Acid in Blood and Tissues

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The chemical methods which have been proposed for the estimation of acetic acid in biological materials are not specific and are, in consequence, of restricted value. Treatment of acetic acid with cupric oxide yields oxalic acid, and this reaction was utilized by Mugdan & Wimmer (1933) for the estimation of acetic acid; but, under their conditions tartaric, glycollic and the lower fatty acids also yield oxalic acid. Pyrolysis of calcium acetate yields acetone, but since the calcium salts of other monocarboxylic acids also yield carbonyl compounds under these conditions, the method of Dulian (1939), which is based on this reaction, is inadequate. A photometric method based on the lanthanum reaction of Kruger & Tschirch (1929) has recently been developed by Hutchens & Kass (1949), but since propionic acid also gives the blue colour their method lacks specificity.

Procedures dependent on the physical properties of acetic acid can be classified into: (i) Distillation methods: Duclaux (1900), Dyer (1917), Olmsted, Whitaker & Duden (1929), McClendon (1944) and

Black (1949). (ii) Partition methods: Behrens (1926), Werkman (1930*a, b, c*, 1931). Friedemann (1938) devised a method which is a combination of both the distillation and partition methods. (iii) Chromatographic methods: Ramsey & Patterson (1945, 1948), Elsdon (1946), Ramsey (1948), Moyle, Baldwin & Scarisbrick (1948) and Peterson & Johnson (1948). Of all these procedures only those involving chromatography can make any real claim either to specificity or to precision.

Acetic acid, however, does differ from the other members of the homologous series in its stability to the potassium dichromate-sulphuric acid reagent; but at the same time it is completely oxidized by treating it with a silver dichromate-sulphuric acid mixture (Simon, 1922*a, b*). Cordebard & Michl (1928) used this difference in behaviour as the basis of a quantitative method for the estimation of acetic acid in known mixtures; their method was adapted to the micro-estimation of acetic acid by Caselli & Ciaranfi (1942). In this procedure acetic acid was separated from its higher homologues by conversion

to the methyl ester and subsequent distillation. The interfering compounds which distilled with methyl acetate were allowed for by treating with potassium dichromate-sulphuric acid prior to oxidation with the silver dichromate-sulphuric acid reagent. This method, whilst useful for known mixtures, was not directly applicable to the estimation of acetic acid in biological materials. It has, however, proved possible to modify the technique in such a way as to permit the photometric estimation of acetic acid in blood and tissues.

METHOD

Principle. Methyl acetate (b.p. 57°) boils at a lower temperature than methanol (b.p. 65°), whereas the methyl esters of the other members of the homologous series have a higher boiling point; consequently by esterifying a mixture of fatty acids in excess methanol and subsequent distillation it is possible to effect a considerable separation of acetic acid from the remainder. After one such distillation and with the relative recovery of acetic acid placed at 100, the recoveries of other acids are as follows: formic, 87; propionic, 63; butyric, 44; valeric, 8; hexanoic, 3; heptanoic, 1. The recovery of formic acid is not quantitative as it is partially decomposed during esterification in the presence of H_2SO_4 . H_2S , produced from cysteine and related compounds, is converted to methyl mercaptan and distils under these conditions. By repeated distillation methyl mercaptan, methyl formate and methyl acetate are separated from the methyl esters of the higher acids, and saponification of the esters in the final distillate yields the sodium salts of H_2S , formic and acetic acids. The first two are readily oxidized by treatment with the $K_2Cr_2O_7$ - H_2SO_4 reagent, whereas acetate is oxidized only in the presence of Ag^+ .

Under standard conditions the extra dichromate used following treatment with $Ag_2Cr_2O_7$ is related to the amount of acetate present. The dichromate may be estimated either photometrically, using a filter absorbing at 450 $m\mu$, or titrimetrically.

Reagents. Methanol (A.R.). Traces of volatile acids are removed by adding 2 g. of NaOH/l. of methanol and distilling from an all-glass apparatus. Conc. H_2SO_4 (sp.gr. = 1.84). NaOH (40%, w/v, and 1.25% w/v). Saturated aqueous solution of $AgNO_3$. 0.05N- $K_2Cr_2O_7$.

Apparatus. (a) Distillation units, seven of which are required. These are constructed of 8 mm. bore tubing and are of the micro-Kjeldahl type as modified by Embden. The ascending limb is 15 cm. long with a bulb of 3 cm. diameter situated 5 cm. above the standard joint. The oblique limb is

20 cm. long and the descending limb 40 cm.; the first 18 cm. of the latter is water-jacketed.

(b) A supply of 50 and 100 ml. Kjeldahl flasks fitted with standard joints. The 50 ml. flasks carry a 12 ml. calibration mark.

(c) Two Schiff-type reflux condensers 80 cm. long and fitted with standard joints.

(d) A Coleman spectrophotometer model S-14 with round cuvettes (19 × 105 mm.) or a Lumetron photoelectric colorimeter model 400-A.

Extraction. Blood (5 ml.) is placed in a centrifuge tube containing 40 ml. of cold methanol, stirred thoroughly with a glass rod and centrifuged at 5000 rev./min. for 5 min. in a refrigerated centrifuge. The supernatant is transferred to a measuring cylinder and the volume noted. It is filtered into a 100 ml. Kjeldahl flask and both cylinder and filter paper are washed with a few ml. of methanol, the washings being added to the Kjeldahl flask.

Tissues. A known weight of tissue (approx. 1 g. when using liver, kidney or muscle) is placed in a cooled glass mortar and ground thoroughly with powdered quartz sand; 5 vol. of cooled methanol are then added and stirred with a glass rod. The mixture is transferred to a centrifuge tube and the mortar washed with a further 5 vol. of cooled methanol and the washings added to the centrifuge tube. The contents are made alkaline with 0.2 ml. of 40% (w/v) NaOH and procedure continued as for blood.

Analytical procedure. The Kjeldahl flask containing the methanolic extract is attached to a distillation unit and the contents boiled gently in order to minimize frothing. When the volume has been reduced to about 3 ml. the flask is disconnected and the contents taken to dryness in a bath of boiling saturated $CaCl_2$, the vapours being removed with a water pump. The flask is then cooled and 15.0 ml. methanol and 1.0 ml. conc. H_2SO_4 added. The ground-glass joint is lubricated with a few drops of conc. H_2SO_4 and the flask attached to a second distillation unit. Methanol (4.0 ml.) is added to a 50 ml. Kjeldahl flask, which serves as a receiver, and which is so placed that the tip of the condenser dips below the surface of the methanol. It is distilled gently until the volume in the receiver is 12 ml.; the tip of the condenser is rinsed with 4.0 ml. methanol which are added to the rinsings in the receiver. During this step the fatty acids present in the extract are esterified and the esters distilled over. The methyl acetate is next separated from the esters of the higher acids by repeated distillation; in all, five such distillations are required for complete separation (Table 1). In each case distillation is stopped when the volume in the 50 ml. Kjeldahl flask is 12 ml. and the tip of the condenser washed down with 4 ml. methanol. The final distillate is

Table 1. Elimination of C_3 - C_7 fatty acids by successive distillations of their methyl esters

Acid	Amount added (mg.)	Amount* (mg.) recovered after					
		1	2	3	4	5	6
No. of distillations ...	...						
Formic	1.00	0.88	0.86	0.90	0.84	0.85	0.90
Acetic	1.00	0.98	1.05	1.02	1.00	1.01	1.03
Propionic	1.00	0.63	0.41	0.29	0.15	0.04	—†
Butyric	1.00	0.44	0.22	0.11	—	—	—
Valeric	1.00	0.08	—	—	—	—	—
Hexanoic	1.00	—	—	—	—	—	—
Heptanoic	1.00	—	—	—	—	—	—

* Mean values of several determinations. Individual determinations ranged within $\pm 5\%$ of the mean.

† — signifies that the fatty acid amount was not measurable.

collected in 4.0 ml. of 1.25 % (w/v) NaOH instead of methanol. A glass bead is added and the mixture set aside for 30 min. to ensure complete hydrolysis of the esters. The contents of the flask are then taken to dryness in the boiling CaCl_2 bath. The dry sodium salts are next treated with the oxidizing agents as follows. 0.05 N- $\text{K}_2\text{Cr}_2\text{O}_7$ (7.0 ml.) is added followed by 7.0 ml. conc. H_2SO_4 , the latter being added in such a way as to moisten the standard joint. The flask is attached to a reflux condenser and the contents boiled for 30 min. When the flask has cooled 4.0 ml. of the contents are removed with a pipette and delivered into a colorimeter tube containing 6.0 ml. water; this sample is called 'K unknown'. Two drops of saturated AgNO_3 and 3.0 ml. conc. H_2SO_4 are added to the remaining liquid in the flask and the contents boiled under a reflux condenser for

Fig. 1. Calibration curve obtained by estimating samples of standard acetic acid solution, dried together with 4.0 ml. of 1.25 % (w/v) NaOH.

1 hr. When cool, a second 4.0 ml. sample is transferred to a colorimeter tube containing 6.0 ml. water; this sample is called 'Ag unknown'. Whilst carrying out the oxidation of the unknown a reagent blank is performed and the two 4.0 ml. samples taken are referred to as 'K blank' and 'Ag blank' respectively. The amount of dichromate present in each sample is then determined spectrophotometrically. If a suitable spectrophotometer is used such as one of those specified it is possible to correct for the blanks directly on the instrument. In all there are three corrections to be made, the 'K blank' and the 'Ag blank' and, in addition, that due to materials present in the distillate which are oxidized by dichromate in the absence of Ag^+ , i.e. formate and sulphide.

If the amount of dichromate taken has been sufficient to oxidize all the acetate present then, by setting the 'Ag blank' at an extinction of 0.4 the 'Ag unknown' will give a reading of between 0.4 and 0.0. If this is the case, the 'K unknown', which corresponds to zero acetate concentration, is set at an extinction of 0.4 and the extinction of the 'K blank' determined. This is equal to or greater than 0.4 depending on the amount of easily oxidizable material (formate and sulphide) present in the sample. The reading of the 'K blank' so obtained is used as the arbitrary zero against which the extinction of the 'Ag unknown' is measured. Fig. 1 gives the calibration curve of the method showing that there is a

linear relationship between acetic acid and extinction over the range 0.0–2.3 mg. acetic acid.

Titrimetric method. If a photometer is not available a titrimetric procedure similar to that of Caselli & Ciaranfi (1942) may be used. In addition to the apparatus and reagents specified for the photometric procedure the following solutions are required:

0.05 N- $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ in 5 % (v/v) H_2SO_4 ,

0.05 N- KMnO_4 .

The extraction and distillation procedures are the same as those outlined above. However, the final distillate, which is collected in 4.0 ml. 1.25 % (w/v) NaOH solution, is treated differently. The distillate is divided into two equal parts each of which is transferred to a 50 ml. Kjeldahl flask. After allowing 30 min. to complete the hydrolysis of the esters the contents of each flask are taken to dryness. To one flask is added 5.0 ml. 0.05 N- $\text{K}_2\text{Cr}_2\text{O}_7$ and 5.0 ml. conc. H_2SO_4 and to the other 5.0 ml. 0.05 N- $\text{K}_2\text{Cr}_2\text{O}_7$, two drops saturated AgNO_3 and 8.0 ml. conc. H_2SO_4 . Each flask is then boiled for 1 hr. under a reflux condenser and, when cool, 5.0 ml. of 0.05 N-ferrous ammonium sulphate added, and the contents titrated with 0.05 N- KMnO_4 . In addition, both a 'K blank' and an 'Ag blank' are determined. When acetate is oxidized under these conditions, 1 ml. of 0.05 N-dichromate is equivalent to 0.375 mg. acetic acid. The amount of acetic acid present in half the sample of blood or tissue taken is equal to $[(A - a) - (K - k)] \cdot 0.375$ mg., where A , a , K and k are the volumes of 0.05 N- KMnO_4 required for the 'Ag unknown', 'Ag blank', 'K unknown' and 'K blank' respectively.

RESULTS

Recoveries of known amounts of acetic acid added to various tissues as determined by the photometric and the titrimetric procedures are given in Tables 2 and 3 respectively; Table 4 gives the results of estimations of the acetic acid content of blood, liver,

Table 2. Recovery of acetic acid estimated photometrically

('Found' values are corrected for acetic acid present in controls.)

Material	Acetic acid	
	Added (mg.)	Found (mg.)
Blood (human)	0.13	0.13
	0.26	0.24
	0.39	0.36
	0.78	0.84
	1.17	1.15
	1.30	1.24
	1.43	1.36
Muscle (rat)	1.95	1.90
	0.37	0.40
Kidney (rat)	0.74	0.72
	0.70	0.72
Kidney (guinea pig)	1.11	1.08
	0.30	0.32
	0.39	0.38
	0.56	0.53
	1.26	1.21
Liver (guinea pig)	0.39	0.39
	0.61	0.58

Table 3. *Recovery of acetic acid estimated volumetrically from human blood*

('Found' values are corrected for acetic acid present in controls.)

Acetic acid	
Added (mg.)	Found (mg.)
0.13	0.14
0.22	0.23
0.25	0.25
0.50	0.52
0.51	0.48
0.53	0.51
0.54	0.58
0.56	0.55
0.63	0.61
1.00	0.94

Table 4. *Acetic acid determinations in blood and tissues*

		Acetic acid found (mg./100 ml. for blood, mg./100 g. for tissues)		No. of determina- tions
Material		Mean	Range	
Blood	Human venous*	4.8	3.2-7.2	10
	Sheep arterial*	7.1	4.6-8.8	3
	Guinea pig arterial†	10	8.2-12.0	3
	Rat arterial†	9	6.1-12.0	3
Liver	Guinea pig†	61	35-98	5
	Rat†	54	40-69	3
Kidney	Guinea pig†	47	27-71	3
	Rat†	35	20-52	3
Muscle	Guinea pig†	23	11-36	3
	Rat†	16	10-22	3

* 6 hr. after feeding. † 1 hr. after feeding.

kidney and muscle. Reid (1950) determined the acetic acid content of sheep blood using the chromatographic methods of Elsdén (1946) and of

Moyle *et al.* (1948), and our figures for sheep blood are in agreement with those obtained by this author. On the other hand, McClendon (1944), in his paper on the micro-estimation of total volatile fatty acids in biological materials, states that the venous blood of both normal humans and dogs is 0.0003N in respect of these acids; our results for acetic acid alone are substantially higher. Unfortunately, data are lacking for the acetic acid content of other tissues using other methods, and we are thus unable to obtain a comparison of our method.

SUMMARY

1. Acetate, extracted from blood or tissues with methanol, is dried and transformed into methyl acetate. By repeated distillations the C_3 - C_7 fatty acid esters are removed. The ester is saponified and the resulting sodium acetate brought to dryness. Formic acid and hydrogen sulphide, which cannot be eliminated in such a way, are oxidized by potassium dichromate-sulphuric acid, the acetic acid remaining unchanged. Then acetic acid is fully oxidized by increasing the sulphuric acid concentration and by adding silver ions. The reduction of dichromate is measured photometrically and the acetic acid is estimated by the difference in the extinction.

2. This method permits the estimation of acetic acid in blood and tissues (0.1-2.3 mg.) with sufficient accuracy for biological purposes. The error is always less than $\pm 10\%$.

3. The concentrations of acetic acid under physiological conditions, in human blood and in the blood, liver, kidney and muscle of the guinea pig and rat are discussed.

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